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Defect of In Vitro Digestive Ability of Polymorphonuclear Leukocytes in Paracoccidioidomycosis

MAURICIO GOIHMAN-YAHR,[1]* ERVIN ESSENFELD-YAHR,[2] MARÍA C. DE ALBORNOZ,[1] LUIS YARZÁBAL,[1] MARÍA H. DE GÓMEZ,[1] BLANCA SAN MARTÍN,[1] ANA OCANTO,[1] FRANCISCO GIL,[3] AND JACINTO CONVIT[1]

Pan American Health Organization Center for Research and Training in Leprosy and Tropical Diseases (CEPIALET), Instituto Nacional de Dermatología[1]; Department of Pathology, Hospital Padre Machado[2]; and Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas,[3] Caracas, Venezuela

Selected functions of polymorphonuclear leukocytes were studied in patients with paracoccidioidomycosis (South American blastomycosis), in healthy control individuals, and in patients with diseases unrelated to paracoccidioidomycosis. Patients with paracoccidioidomycosis were also evaluated by standard immunological techniques. Phagocytosis and digestion of *Paracoccidioides brasiliensis* yeastlike cells in vitro was estimated by an original method. It was based on the appearance of phagocytosed *P. brasiliensis* in preparations stained by a modification of the Papanicolaou method and examined with phase-contrast optics. Interpretation of such findings was confirmed by electron microscopy. Two strains of *P. brasiliensis* were used. Strain 8506 was freshly isolated from a patient. Strain Pb9 was known to be nonpathogenic and to have a peculiar cell wall composition. Yeastlike cells of the Pb9 strain were digested significantly better than those of strain 8506. A higher number of leukocytes per fungus cells led to a higher proportion of digested *P. brasiliensis*. Leukocytes from patients with paracoccidioidomycosis phagocytosed the fungus in a normal way, but had a significant lower ability to digest it in vitro. When individual cases were analyzed, there was an excellent correlation between clinical evolution and digestive ability of polymorphonuclear leukocytes. There was good correlation between both of these and immunological parameters. Leukocytes from all groups behaved comparably in tests of general leukocyte function and in their abilities to kill and digest *Candida albicans*. Our results indicate that, as a group, polymorphonuclear leukocytes from patients with paracoccidioidomycosis had a significant, rather specific, defect in their in vitro digestive capacity against phagocytosed *P. brasiliensis*. There was also an inverse correlation between strain pathogenicity and its susceptibility to in vitro digestion by polymorphonuclear leukocytes. Our findings are concordant with and relevant to clinical reality.

Paracoccidioidomycosis (South American blastomycosis) is a granulomatous disease. It occurs when *Paracoccidioides brasiliensis* grows in the tissues of infected, susceptible individuals. Many organs or structures may be affected. Among these are lungs, mucosae, lymph nodes, spleen, adrenals, and other viscera. Clinical features, evolution, and prognosis are not homogeneous. Diverse clinical forms have been described (2, 16). Some patients may deteriorate swiftly and die if untreated. Others are able to localize the disease. There is evidence of subclinical infections (4) and of very long incubation periods (3). Although the lungs are affected and the fungus may be isolated from the oral mucosa and sputum, person-to-person contagion and accidental laboratory disease have not been demonstrated to our knowledge. Accidental inoculation in the laboratory has been reported (31), but with no clinical illness. The organism is able to produce clinical disease when inoculated into animals, and it has been isolated from the soil (9). Soil isolation is not easy with available techniques.

Paracoccidioidomycosis tends to be circumscribed to certain geographic areas within given countries (5). Nonetheless, intradermal testing has indicated that exposure and sensitization to the organism are much more common than clinical disease (1, 21, 22, 24). This is also true for other deep mycoses (39).

In paracoccidioidomycosis, as in other deep mycoses, there is a need to determine factors that may explain the increased susceptibility of certain individuals. It has been shown that patients with disseminated disease have high levels

of circulating antibodies, but do not develop detectable delayed hypersensitivity against *P. brasiliensis* (12, 13, 41). An equivalent phenomenon takes place in coccidioidomycosis.

We wished now to explore the role of polymorphonuclear leukocytes (PMNs), particularly since in some granulomatous diseases the function of such cells is abnormal. In some instances the abnormality takes place in a discrete identifiable step of the metabolic processes that would normally lead to the killing of ingested microorganisms (e.g., granulomatous disease of childhood [23]). In other cases, a wider spectrum of neutrophil functions is altered (7).

As will be shown, we found a significantly lower ability of PMNs from patients with South American blastomycosis to digest *P. brasiliensis* in vitro. Overall neutrophil function and digestive capacity against *Candida albicans* did not differ significantly from that of cells from control individuals.

Clinical evolution, in vitro digestive capacity of PMNs against *P. brasiliensis,* and kind of immune response seemed to be related. A favorable clinical evolution correlated with good in vitro digestive abilities and with the absence of detectable circulating antibodies and presence of positive intradermal tests against *P. brasiliensis* antigens.

Our results point to the importance of relatively specific phagocytic cell defects in the pathogenesis of granulomatous diseases produced by intracellular organisms.

MATERIALS AND METHODS

Individuals tested. Three groups of 16 persons each were explored. Groups were comparable concerning age and sex. Healthy controls were volunteers recruited from personnel of the Instituto Nacional de Dermatología (physicians, medical students, technicians, and maintenance personnel). Control patients with diseases unrelated to paracoccidioidomycosis were individuals hospitalized in our wards for a variety of conditions; two patients in this group were ambulatory. Persons with paracoccidioidomycosis came from diverse sources; most were hospitalized in our wards or outpatients in our clinics, and others were referred from the Hospital Simón Bolivar, a predominantly chest hospital, or from physicians in different areas of Venezuela. Most patients were studied before starting specific therapy or while in remission, when no therapy was indicated. In other cases, specific treatment was stopped for at least 2 weeks before tests. In two cases this was not possible; treatment was discontinued for at least 3 days, and the serum of the patient was not used in the in vitro assays (see below).

Except for the above, persons tested did not receive any drug known to alter PMN function or that would affect *C. albicans* or *P. brasiliensis* viability.

All tested individuals went through a protocol that included a clinical history, physical examination, routine laboratory testing (complete blood count, blood chemistry, urine examination), and chest radiographies, as well as other tests required for the diagnosis of the disease present if any. Diagnosis of paracoccidioidomycosis was done by biopsy, by observation of the fungus in direct microscopic examination, and by culture. Whenever necessary, patients had additional tests such as respiratory and adrenal function tests. Initially, patients were classified according to the classification of Giraldo et al. (16). For our purposes, however, it was better to simply divide them according to the overall evolution. Evolution was "favorable" when the patients were clinically healed by treatment and were currently without medication. "Borderline" was used to describe patients with a disease that progressed rather slowly but had a tendency to become widespread unless treatment was carried out. "Unfavorable" was used to describe patients with rapidly progressing disease with dissemination.

Immunological testing. Patients with paracoccidioidomycosis were tested intradermally using antigens prepared according to the Fava-Netto (11, 14) and Restrepo-Moreno et al. (35) methods. Sites were read at 4, 24, and 48 h after injection. The criterion for positivity was an infiltration of a diameter equal to or exceeding 5 mm at 24 or 48 h. Strong reactions had indurations exceeding 10 mm in diameter. Sera were tested by double diffusion in agar as described by Yarzábal et al. (42) and by immunoelectrophoresis as reported by Yarzábal (40). Complement fixation tests using *P. brasiliensis* yeastlike- and filamentous-phase antigens were kindly done by Ladislao Pollak (Instituto Nacional de Tuberculosis J. I. Baldó, Caracas, Venezuela). Although titers were not identical against both antigens, they showed good correlation.

Patients were broadly classified according to their immunological response as follows. "Favorable" defined positive intradermal tests (to one or both antigens) and negative serological tests. "Borderline" was used to define patients who had a weakly positive intradermal test with positivity in one or two serological tests, and complement fixation titers were lower than 1:32. "Unfavorable" defined negative intradermal tests, positive serological tests, and a complement fixation titer of 1:32 or higher.

As happened with clinical evolution and PMN digestive abilities, some patients defied strict inclusion in any of the above three categories. Intermediate slots were created for them (Table 3). The existence of two "polar" responses and an intermediate spectrum of immune responses has been emphasized previously (30, 31).

Microorganisms used. Stock cultures of *C. albicans* were kept in Sabouraud agar slants (Difco) at room temperature (around 22°C in Caracas). For experiments, 50-ml Erlenmeyer flasks containing 15 ml of Sabouraud liquid medium (Difco) were inoculated and kept at room temperature as stationary cultures (with infrequent manual agitation). Suspensions were harvested after no less than 4 days and no more than 7 days of incubation.

Two strains of *Paracoccidioides brasiliensis* were used in all cases. Strain Pb9 was kindly supplied by L. and G. San Blas (Instituto Venezolano de Investigaciones Científicas, Venezuela). This is a nonpathogenic

strain with a peculiar cell wall (36, 37). Strain 8506 was isolated by us from a case of paracoccidioidomycosis. We were able to test the leukocytes of two patients against the strain of *P. brasiliensis* causing their disease. Stock cultures were kept in agar slants (brain heart infusion agar, (Difco) at 36.5°C. For growth in liquid medium, 15 ml of brain heart infusion (Difco) was put in 50-ml Erlenmeyer flasks plugged with gauze and cotton. The medium used for the initial inoculum had 50 μg of gentamicin (reagent grade; Schering) per ml. Flasks were put in a reciprocating water bath shaker (Eberbach Corp., Ann Arbor, Mich.) at 34°C (growth of the yeastlike phase is better at that temperature, as has been reported [28]). Fresh flasks, without antibiotic, were inoculated every 3 to 4 days thereafter (late log phase). Growth curves were initially estimated by direct particle count.

Tests of neutrophil function. (i) Nitro Blue Tetrazolium. Reduction of Nitro Blue Tetrazolium in vitro was estimated by the percentage of neutrophils that showed dark-blue formazan precipitates in their cytoplasm after incubation with Nitro Blue Tetrazolium in vitro (formazan-positive cells). We employed a modification of the technique described by Matula and Paterson (29) as reported by us (19, 20), with simultaneous in vitro activation with endotoxin (lipopolysaccharide W, *Escherichia coli* O26:B6; Difco). Cover slips were mounted, and at least 100 neutrophils were counted. Only intact cells were taken into consideration. Results were expressed as percentage of formazan-positive cells.

(ii) Peroxidase stains. Stains were done according to a modification of the method of Beacom (4), which in itself is a modification of Goodpasture's technique. Slides were counterstained with Wright-Giemsa. A parallel Wright-Giemsa-stained smear was used to determine relative proportions of PMNs and monocytes in the subject's peripheral blood. Slides were evaluated according to an arbitrary point scale: 0, no dark granules in PMNs, only background stain; 1, a light scattering of dark granules in the cytoplasm, both cytoplasm and nucleus easily identified; 2, dark granules throughout the cytoplasm, the nucleus may be identified; 2.5, very many and coarse dark granules in the cytoplasm, and the nucleus is still detected although its exact shape cannot be determined; 3, very many and coarse dark granules in the cytoplasm, covering the nucleus so that it cannot be identified. In a satisfactory preparation, lymphocytes would not show dark granules by this method.

Isolation of peripheral blood leukocytes. A 40-ml sample of fasting blood was drawn from a peripheral vein by means of a no. 19 butterfly infusion set. Blood was heparinized immediately (10 U of sodium heparin [Lilly] per ml) and mixed with dextran (6% Macrodex Pharmacia in saline; mean molecular weight, 70,000). One milliliter of dextran was used per 9 ml of blood. The mixture was sedimented at 36.5°C for 30 to 45 min (additional blood was processed to obtain fresh autologous serum, or else fresh frozen autologous or frozen AB Rh-positive pooled serum [in patients under treatment] was employed). After sedimentation of the blood-dextran mixture, the upper layer was drawn off by means of a syringe with a hematocrit needle (pipette for Wintrobe tube no. 16; Clay Adams). This layer was centrifuged (800 rpm for 10 min in a GLC-2B General Laboratory Centrifuge; radius, 16.41 cm), washed twice in Hanks balanced salt solution (GIBCO) with 10 U of sodium heparin per ml and 50 μg of gentamicin per ml (reagent grade; Schering) (HHG), and finally suspended in 3.2 ml of HHGFCS (HHG plus 20% fetal calf serum [GIBCO]). Leukocytes were counted using standard counting solutions and a hemacytometer. Suspensions were adjusted with HHGFCS to contain 10^7 PMNs per ml. No hypotonic lysis was done. The viability of PMNs as estimated by the erythrosin B method (32) was about 98%.

Fungal suspensions. A 5-ml sample of *C. albicans* in Sabouraud liquid medium was centrifuged in a GLC-2 centrifuge at 1,500 rpm for 10 min and washed twice in sterile saline. Yeasts were counted in a hemacytometer, and suspensions were adjusted to contain 10^7 yeast cells per ml of HHGFCS. Viability of *Candida*, estimated by the methylene blue test (25), was above 95% before incubation.

P. brasiliensis. Suspensions of the two strains were processed simultaneously and in the same way. The procedure has been reported (17; M. Goihman-Yahr, L. Pine, M. C. Albornoz, L. Yarzábal, A. Ocanto, M. E. Gómez, and J. Convit, Acta Cient. Venez. **28**(Suppl. 1):118–119, 1977). Briefly, 6 ml of *P. brasiliensis* in brain heart infusion liquid medium was obtained under sterile conditions. This volume was centrifuged (1,500 rpm for 10 min in a GLC-2 centrifuge), and cells were washed once in sterile saline. Single-cell suspensions were obtained by means of ultrasound as follows. Portions of the washed suspensions (1 ml) were put into sterile polystyrene tubes (12 by 75 mm). The tubes were kept standing in a specially designed holder, surrounded by ice water. Sonication was done with a 185 WD Branson sonifier (Sonifier cell disruptor model W185 D; Heat Systems Ultrasonics, Inc., Plainview, N.Y.) using a specific microtip (special stepped microtip, Heat Systems Ultrasonics) previously sterilized by flaming ethanol. The ensemble was held in a specially designed holder that prevented lateral tilting and also fixed the position of the tip vertically. Suspensions were sonicated at low intensity (scale 1 of the apparatus) for exactly 20 s. After vibration, contents of the plastic tubes were pooled and washed twice more with saline. Yeastlike cells were counted in a hemacytometer using phase-contrast optics (DLL Nikon objectives with a Nikon phase contrast turret condensor). Cells were centrifuged once more and suspended in HHGFCS. Concentrations were adjusted to either 10^7 yeast cells per ml (full strength) or 2×10^6 cells per ml (1/5).

Latex suspensions. Latex particles (Latex 0.81; Difco) were suspended 1:60 (vol/vol) in HHGFCS.

Mixtures. Incubation was done in sterile polystyrene tubes (12 by 75 mm). Each received a total of 1 ml as follows: (i) 0.25 ml of PMNs (10^7/ml in HHGFCS); (ii) 0.25 ml of particles in HHGFCS (*C. albicans*, 10^7/ml, or *P. brasiliensis*, full or 1/5 strength, or latex particles); (iii) 0.25 ml of human serum (sterilized by pressure filtration through membranes [Millipore], 0.45-μm mean pore size) (serum was usually fresh or frozen autologous; occasionally, pooled frozen AB Rh-positive serum was used); and

(iv) 0.25 ml of HHG. Control tubes received 0.25 ml of HHGFCS instead of the leukocytes.

Tubes were put in a rotating holder (Multipurpose Rotator 150V; Scientific Industries, Inc., Springfield, Mass.) at 30 rpm and incubated at 37°C. Tubes containing latex particles were incubated for 1 h. Those containing *P. brasiliensis* or *C. albicans* were incubated for 1 h and 2.5 h. After incubation, tubes were processed as follows. For phagocytic index of latex particles, 2 drops of the incubated suspension was added, together with 6 drops of HHGFCS, to the preparative chamber of a Shandon-Elliott cytocentrifuge (Shandon Elliot Cytospin SCA-0030, Shandon Southern Instruments Ltd.). The chambers were rotated at 800 rpm for 10 min. Duplicate droplets were air dried and stained with Wright-Giemsa. They were examined unmounted, with phase-contrast oil-immersion optics. A reticulated eyepiece (Bausch & Lomb micrometer disk) was useful. The number of intracellular latex spherules was counted in 100 neutrophils and at least two droplets. The phagocytic index was thus determined.

For the phagocytic index of *C. albicans* and *P. brasiliensis* the same method was employed, except that in the case of *Candida,* mounted, Wright-Giemsa-stained preparations were examined with standard optics. In the case of *P. brasiliensis,* full- or 1/5-strength tubes were proccessed as described. Droplets were stained by our variation of the Papanicolaou method (17), and mounted droplets were examined with phase-contrast optics for determination of the phagocytic index after h 1 of incubation.

Killing assay against *C. albicans* was done according to the method of Lehrer and Cline (26). The percentage of dead (i.e., blue-colored) yeasts was estimated after 1 h of incubation in samples from test and control tubes. Net killing was the difference between the percentage of blue-colored *Candida* in leukocyte-containing and in control tubes.

Digestive ability of PMNs against *C. albicans* was estimated by the method of Lehrer (25). After 2.5 h of incubation, percentage of "ghosts" was determined with standard optics, counting at least 200 intracellular organisms in at least two droplets.

Digestive ability of PMNs against *P. brasiliensis* was estimated according to our procedure using a modification of the Papanicolaou staining method (17). Briefly, the leukocytes of each individual were tested against each of the two strains of *P. brasiliensis* (Pb9 and 8506) at two different concentrations (full and 1/5 strength and at two different time intervals (1 h and 2.5 h). Thus, at least eight different values were obtained for each subject; in two patients we also tested their leukocytes against the respective strain causing their disease. Droplets were obtained as already described and processed as reported by us (17). At least two droplets and no less than 200 phagocytosed *P. brasiliensis* yeast cells were examined. Unaltered *P. brasiliensis* yeast cells showed a green cell wall and a brownish-orange patterned protoplasm. Digested *P. brasiliensis* cells had a "ghostlike" appearance ("G" cells): the cell wall surrounded an empty, faintly green space. Partially digested organisms showed a homogeneous, faintly colored protoplasm or only a rim of patterned protoplasm surrounding a greenish empty space. The distinction between intact and G cells was much facilitated by the use of phase-contrast optics. The results obtained were expressed as percentage of G cells (totally or partially digested). Selected samples were processed for electron microscopy as follows. After incubation, the culture medium was removed by centrifugation, and the pellet was suspended and fixed for 3 h in 2% (vol/vol) glutaraldehyde in cacodylate buffer (pH 6.7; 0.1 M). The pellet was then washed three times in the cacodylate buffer and postfixed in osmium tetroxide (2%, wt/vol) for 2 h. After one wash in buffer the suspension was centrifuged at 4,000 × *g* for 5 min, suspended in 2% agar at 50°C, and immediately centrifuged at 5,000 × *g*. The solidified agar was removed from the tube, and the suspension zone was cut into 1-mm^3 pieces. These were dehydrated in an ethanol series and embedded in Epon 812 (1 A: 1 B) mixture as described by Luft (27). Thin sections were cut with a diamond knife in a Porter-Blum MT-1 ultramicrotome and stained with aqueous uranyl acetate (2%, wt/vol) for 10 min at 60°C and with lead citrate for 10 min at room temperature. Sections were examined by means of a JEOL JEM-100 B electron microscope at 80 kV.

Statistical analysis was performed whenever pertinent by Frederick Dorey (Department of Biomathematics, University of California, Los Angeles) using a continuous normal theory, repeated-measures analysis of variance model. A Biomedical Computer Program P Series (Dixon and Brown [10]) was employed.

Studies reported here were extended for more than 1 year. We processed one or only a few individuals per week. Care was taken to insure that distribution of tested individuals from different groups would be comparable in time. Series were started and ended in similar dates for all groups. Whenever possible, in a given week, cells from patients with paracoccidioidomycosis and from control individuals were processed simultaneously or consecutively, depending on the test carried out.

RESULTS

Table 1 summarizes the results of general PMN function tests in the three experimental groups. It also includes killing and digestion assays against *C. albicans*. It is evident that PMNs from all groups were equally capable of phagocytosing latex and *Candida*. They were equally capable of being activated by endotoxin and had essentially the same peroxidase activity as estimated by the method employed. (One patient with paracoccidioidomycosis had a very doubtful peroxidase activity when tested initially. A repeat test was negative [0]. The patient was 77 years old; his neutrophils had a normal *Candida*-killing and *Candida*-digesting ability. His clinical evolution, immunological responses, and in vitro digestive abilities of his leukocytes against *P. brasiliensis* were borderline.) Table 1 also shows that paracoccidioidomycosis did not, by itself, induce spontaneous activation of circulating neutrophils above levels of control groups, as estimated by the Nitro Blue Tetrazolium tests.

Mean killing capacity against *Candida* of leukocytes from patients with paracoccidioidomycosis, as well as their digestive ability against this fungus, was somewhat lower than that of control groups. The difference was not statistically significant ($P > 0.05$).

Table 2 indicates that phagocytosis of *P. brasiliensis* by PMNs was essentially similar in all groups. Phagocytic indexes were close to values theoretically expected from the relative proportions of neutrophils and *P. brasiliensis* cells.

The central issue of this paper is the digestive ability of PMNs against *P. brasiliensis* and the role played by diverse variables. Our staining method correlated well with ultrastructural features. Figure 1 shows phagocytosed fungi and their appearance when still intact or in variable degrees of digestion. As already indicated by light microscopy, the cell wall persisted while protoplasm was being digested.

TABLE 1. *General function tests and anti-Candida activity in PMNs*

Group	NBT/saline[a]	NBT/endotoxin[a]	Peroxidase stain	PI[b] (Latex)	PI (*Candida*)	Killing (*Candida*)[c]	Digestibility (*Candida*)[d]
Healthy control	31 (13–70)[e]	76.69 (51–92)	2.93 (2.5–3)	6.08 (1.88–13.22)	1.13 (0.3–2.19)	10.67 (0.55–18.58)	17.77 (5.24–28.98)
Unrelated disease control	25.75 (5–61)	65.13 (10–92)	2.84 (2–3)	4.99 (0.17–12.24)	1.14 (0.59–2.68)	8.02 (−2.71[f]–19.73)	18.86 (16.36–40.48)
Paracoccidioidomycosis	23.88 (3–45)	70.38 (28–92)	2.81 (0[g]–3)	7.34 (0.36–16.54)	1.37 (0.39–2.43)	7.13 (−2.97[f]–17.02)	12.90 (5.55–31.08)

[a] Mean percentage of F.P. cells. NBT, Nitro Blue Tetrazolium.
[b] PI, Phagocytic index.
[c] Values shown are "net killing" (difference between the percentage of blue-colored *Candida* in leukocyte-containing and control tubes).
[d] Percentage of digested (ghostlike) phagocytosed *C. albicans*.
[e] Figures in parentheses indicate the range.
[f] The serum of one patient in each of these groups had high killing ability against *Candida* even after repeated testing; there was no relation to any medication.
[g] One patient showed no peroxidase activity. See text for details.

TABLE 2. *In vitro activity of PMNs against P. brasiliensis*

Strain	Group	Phagocytic index		Digestive capacity[a]			
				FS		1/5	
		FS[b]	1/5[c]	1 h[d]	2.5 h[d]	1 h[d]	2.5 h[d]
8506	Healthy control	0.86 (0.49–1.19)	0.23 (0.12–0.34)	24.04 (9.38–52.61)	39.24 (8.07–61.65)	44.42 (27.9–59.42)	57.83 (32.85–71.43)
	Unrelated disease control	0.93 (0.70–1.21)	0.29 (0.15–0.49)	23.75 (6.70–41.50)	39.00 (5.33–56.28)	39.27 (21.31–62.93)	50.12 (17.29–65.32)
	Paracoccidioidomycosis	1.00 (0.58–1.87)	0.28 (0.14–0.54)	18.78 (5.50–34.29)	21.60 (8.57–43.69)	26.35 (6.76–49.01)	28.20 (6.31–51.23)
Pb9	Healthy control	0.88 (0.58–1.14)	0.23 (0.14–0.42)	37.69 (12.56–66.16)	47.71 (30.59–70.71)	57.43 (40.00–68.27)	63.23 (50.32–77.67)
	Unrelated disease control	0.90 (0.60–1.42)	0.30 (0.14–0.46)	37.48 (7.97–54.15)	51.76 (19.1–66.98)	53.99 (27.49–69.46)	63.38 (47.66–78.33)
	Paracoccidioidomycosis	0.99 (0.70–2.14)	0.28 (0.16–0.56)	25.73 (8.37–49.76)	32.69 (12.71–53.40)	38.67 (10.14–60.82)	40.92 (7.02–64.18)

[a] Mean percentages of G cells; parentheses indicate the range.
[b] FS, Full strength; i.e., one yeastlike cell per PMN in the incubation mixture.
[c] 1/5, One yeastlike cell per five PMNs in the incubation mixture.
[d] Length of time of in vitro incubation.

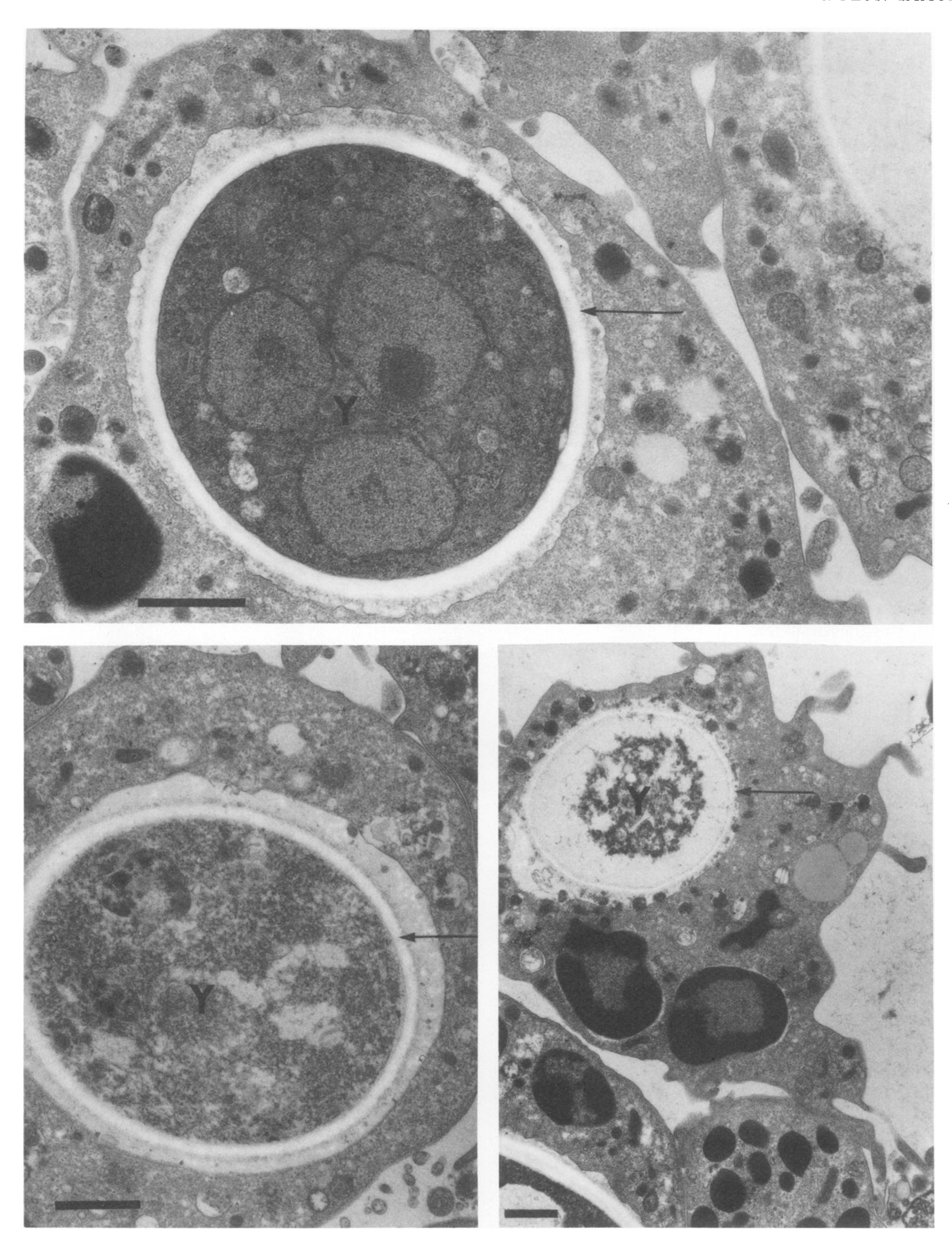


FIG. 1. *Progression of intracellular digestion of phagocytosed P. brasiliensis yeastlike phase (Y) by neutrophils in vitro. The cell wall (arrows) tends to persist while the protoplasm is progressively digested. Bars, 1 μm.*

Table 2 summarizes the results obtained. Statistical analysis of these data indicated the following. (i) PMNs from patients with paracoccidioidomycosis had significantly lower digestive abilities than PMNs from either of the control groups, at all times and all intervals tested ($P < 0.001$). There were no significant differences between abilities of PMNs from both control groups. (ii) The lower concentration of fungi (i.e., higher relative proportion of PMNs) resulted in significantly higher digestion of *P. brasiliensis* ($P < 0.001$). (iii) Digestion of yeast-

like cells from the nonpathogenic (Pb9) strain was significantly higher for all groups than that of cells from the 8506 strain ($P < 0.001$). (iv) The longer incubation interval (2.5 h) resulted in a significantly higher digestion ($P < 0.001$). (v) There was a significant group-time interaction. That is, the digestive capacity for both control groups at the longer interval showed a greater increase (when compared to the shorter interval) than that observed in the group of patients with paracoccidioidomycosis ($P < 0.001$). In other words, the line that would represent the percentage of G cells as a function time in both control groups would have a significantly steeper slope than that of the paracoccidioidomycosis group. (vi) There was a significant ($P < 0.001$) dose-time interaction. That is, for the higher concentration of fungi (full strength), the mean difference between the proportion of G cells at the two incubation times was significantly greater than this difference for the lower concentration of fungi (1/5).

As stated previously, the experiments took place during a period that exceeded 1 year. Strain 8506, originally pathogenic, had therefore many generations of in vitro growth at the end of this study. We compared the mean proportion of G cells in the first eight individuals tested of each group (for every concentration and interval) with that of the last eight individuals. The mean proportion of G cells was significantly higher ($P < 0.05$) in the last eight individuals when normal leukocytes were tested against *P. brasiliensis* 8506. This did not occur when leukocytes from patients with paracoccidioidomycosis were tested. This may indicate some loss of strain 8506 virulence detected by normal neutrophils.

Neutrophils from two patients were tested also against isolates of their own *P. brasiliensis*. Proportions of G cells were within the range observed with strain 8506 or Pb9. One patient digested yeastlike cells better from his own isolates than from those of strain 8506. The clinical evolution in this particular case related better with digestive properties against his own isolates.

Results obtained in patients with paracoccidioidomycosis were not homogeneous. PMNs from some individuals were able to digest *P. brasiliensis* relatively well. PMNs from other subjects were very inert. We correlated clinical evolution of each individual case with proportion of G cells and with outcome of serological tests and results of intradermal testing. Given the size of the sample, a statistical analysis was not feasible. Nevertheless, a qualitative analysis was possible. There was an excellent correlation between clinical evolution and digestive properties of PMNs (Table 3). There was a good correlation between both of these and immunological parameters.

The following two cases are illustrative. Subject 14 is a 56-year-old woman in good general health except for psoriasis en plaques. In 1960 she had brain surgery for what was initially thought to be a cerebellar tumor. The growth proved to be a paracoccidioidal granuloma of the vermis cerebelli. No other lesions were found at that time. She was treated initially with amphotericin B and then with Lederkyn for 2 years. After that time she discontinued therapy. Her general physical examination was normal in 1978. A chest X ray was normal. She had no complement-fixing antibodies and had positive intradermal tests (Table 4). The digestive ability of her PMNs against *P. brasiliensis* was com-

TABLE 3. *Correlation between in vitro digestive abilities of PMNs, clinical evolution, and immunological response in patients with paracoccidioidomycosis*

Patient	Clinical evolution[a]	PMN digestive ability[a]	Immune response[a]
1	UNF/BOR	UNF/BOR	UNF/BOR
2	UNF	UNF	UNF
3	BOR	UNF[b]	BOR
4	BOR	BOR	BOR
5	BOR	BOR	FAV
6	UNF	UNF	BOR
7	BOR	FAV/BOR	FAV
8	BOR	BOR	BOR
9	UNF	UNF	UNF
10	BOR	BOR	UNF/BOR
11	BOR	BOR	BOR
12	UNF	UNF	BOR/UNF
13	UNF	UNF	UNF
14	FAV	FAV	FAV
15	BOR/FAV	BOR/FAV	BOR/FAV
16	UNF	BOR	UNF

[a] FAV, Favorable: in vitro digestive abilities against *P. brasiliensis* 8506 and Pb9 comparable or above mean of control groups. Positive intradermal tests. Negative serological tests. Good response to therapy, in remission without treatment. BOR, Borderline: in vitro digestive ability against *P. brasiliensis* 8506 and Pb9 at the mean level of the paracoccidioidomycosis group. Weakly positive intradermal tests. Some positive serological tests. Complement fixation titers < 1:32. Slow progressive disease. Tends to spread in the absence of specific therapy. UNF, Unfavorable: in vitro digestive abilities against *P. brasiliensis* 8506 and Pb9 well below mean of the paracoccidioidomycosis group. Negative intradermal tests. Positive serological tests. Complement fixation titers ≥ 1:32. Rapidly progressing disease with dissemination.

[b] Digestive abilities of this patient's PMNs against yeastlike cells of the isolate causing his disease were BOR.

TABLE 4. *In vitro digestive capacities against P. brasiliensis of PMNs from two selected patients compared with the means of the healthy control and paracoccidioidomycosis groups*

Subject	Strain 8506 (% G cells)				Strain Pb9 (% G cells)				Complement fixation[a]	
	FS[b]		1/5		FS		1/5		F	Y
	1 h	2.5 h	1 h	2.5 h	1 h	2.5 h	1 h	2.5 h		
Paracoccidioidomycosis	18.78	21.60	26.35	28.20	25.73	32.69	28.67	40.92	1/55	1/49
No. 14	34.29	43.69	49.01	46.46	47.89	46.33	60.70	53.70	0	0
No. 13	5.58	8.57	13.54	10.27	8.37	10.73	10.28	7.02	1/256	1/256
Healthy control	24.04	39.24	44.42	57.83	37.69	47.71	57.43	63.23	0	0

[a] Titer of complement fixation against *P. brasiliensis* filamentous phase (F) and yeastlike phase (Y).
[b] FS, Full strength; see Table 2, footnote *b*.

parable to that of normal neutrophils. Subject 13 is a 50-year-old man. At the time of our test he was hospitalized at the Simon Bolívar Hospital in very poor general health. This was his third hospital admission. He had had treatment with sulfonamides and amphotericin B in 1973 and 1975 with initial improvement, but had relapsed even when taking medication. At the time of our tests he had lesions in the oral mucosa and diffuse bronchopneumonic infiltrates in both lungs. *P. brasiliensis* was isolated from the sputum. Table 4 indicates that he had a highly positive complement fixation titer and that his PMNs digested *P. brasiliensis* very poorly in vitro. Percentage of G cells was far below the mean of the proportion found in patients with paracoccidioidomycosis.

DISCUSSION

Our results show that PMNs from patients with paracoccidioidomycosis have a significantly lower capacity to digest in vitro viable yeast-phase *P. brasiliensis* than PMNs from normal individuals or from patients with unrelated diseases. This difference became more marked with increasing time of incubation and was more manifest when higher proportions of PMNs per fungus cell were employed. There was no overall deficiency (detectable by the tests used) in the general functions of neutrophils from patients with paracoccidioidomycosis. Percentage of formazan-positive cells in neutrophils incubated in Nitro Blue Tetrazolium-saline (Table 1) was high in some individuals from all groups. In healthy controls our base-line levels were somewhat higher than those reported from developed countries. If all other requirements in a given individual (as outlined in Materials and Methods) were fulfilled, a high resting percentage of formazan-positive cells was not a reason for exclusion. To do otherwise would have introduced bias. Yeastlike cells from a nonpathogenic, cell wall-defective strain of *P. brasiliensis* were digested significantly better than those from a more recent isolate, by PMNs from individuals of all groups. Nonetheless, neutrophils from patients with paracoccidioidomycosis were significantly less competent to digest even cells of this strain than PMNs from individuals from control groups. This digestive deficiency was relatively specific because it did not extend to abilities to kill and digest *C. albicans*. Admittedly, other species of fungi and other organisms were not tested.

There was a very good correlation between clinical features and digestive abilities of leukocytes. Size of infecting dose may be critical, as suggested by our results.

There were important individual differences even between cells from normal subjects when their capacities were tested. This is not an unusual finding when destructive activities of phagocytic cells are tested against intracellular pathogens. We obtained results resembling these' when digestive capacities of guinea pig peritoneal macrophages were tested in vitro against *Listeria monocytogenes* (18).

We did not determine whether the impairment in digestive capacity of PMNs from patients with paracoccidioidomycosis was temporal or permanent, or whether the ability to digest *P. brasiliensis* could have a prognostic value in uninfected populations or in relatives of patients. This should be explored in the future.

From the design of the experiments it is clear that factors other than those intrinsic to the PMN could play a role. Serum did not seem to be a factor, because phagocytosis of the fungus was unimpaired and in two cases AB Rh-positive serum did not alter the findings. Nonetheless, the eventual role of serum was not expressly explored in this paper. There is evidence that circulating factors may have a role in the pathogenesis of paracoccidioidomycosis and other deep mycoses (6, 33). The action of lymphocytes was not studied. It is likely that their effect in vivo would be more important than that in the current short-term in vitro experiments.

Even in the most active population of PMNs, only a fraction of ingested fungi were digested. Sixbey et al. (38) found a similar phenomenon in *Histoplasma capsulatum* ingested by PMNs. Lehrer (25), we ourselves, and many others found this to occur in phagocytosed *C. albicans*. A very important factor is the relative proportion of PMNs and fungal cells. It is likely that a greater proportion of PMNs or a continuous influx of such cells, as it should occur in vivo, would lead to a much greater, or even total, destruction of a given inoculum of *P. brasiliensis*.

Our results contradict those of Restrepo et al. (34). Their experimental design was quite different from ours (it was based on dye-exclusion tests to detect killing of *P. brasiliensis*). As previously stated (17), we have not found methods using vital dyes to be reliable when tested with *P. brasiliensis*.

Our results indicate the importance of a phagocytic cell defect in the pathogenesis of paracoccidioidomycosis. This defect has some features of specificity. The existence of such dysfunction opens the way for further study not only in paracoccidioidomycosis, but also in other granulomatous diseases due to live organisms. It may be suggested that whenever PMNs are not able to digest a given intracellular microorganism well, a granulomatous response takes place. If the microorganism is not usually digested by the PMNs (and the reasons for this are not always well understood), this granulomatous response is a "normal" feature of the disease produced by the pathogen (e.g., leprosy, tuberculosis [8]). If only PMNs from very few patients are unable to kill and digest the microorganism, granuloma formation is an extremely ususual feature and the clinical form of the disease is individualized (e.g., *Candida* granuloma, chronic granulomatous disease of childhood). It may well be that subtler and relatively restricted digestive deficiencies in PMNs or other phagocytic cells) permit the appearance of certain granulomatous diseases (such as paracoccidioidomycosis or perhaps coccidioidomycosis) in a minority of the individuals exposed. Since digestive abilities are relative, a massive inoculum would probably infect almost everybody, whereas a very small inoculum could be dealt with by almost anybody.

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